Minimum **I**nformation for **T** regulatory cells (**MIT**REG)

Introduction

The purpose of this document is to enable the description of the generation of T regulatory cell (Treg) products for therapeutic application or experimental usage. It was designed to suit reports using endogenous, induced, antigen-specific and polyclonal freshly isolated and expanded Tregs.

This document is split into four sections, each describing a different aspect of the process. Not all sections will be relevant to all Treg products.

Information in some sections of this document may be covered by other Minimum Information documents, or defined vocabularies. For example, flow cytometry is described in MIFlowCyt[1], microarray data by MIAME[2], T-cell assays by MIATA[3] and production of standardized tolerogenic antigen-presenting cells by MITAP[4]. Authors are encouraged to use these resources as appropriate.

Use of Terminology

The key words "**must**", "**should**", and "**may**" in this document are to be interpreted as follows:

must: This word means that the information is an absolute requirement. Failure to provide this information is in strict violation of the specification.

EXAMPLE: The species and the source of the cell material are required for all experiments.

should: This word means that there may exist valid reasons for particular protocols to not provide this data, but that this data needs to be provided if it is relevant to the protocol.

EXAMPLE: If the Tregs were generated or enriched using an antigen then this must be described, although there may be protocols where polyclonal Tregs are applied.

may: This word means that the data is optional, and does not need to be included but can be provided.

EXAMPLE: The health or age of the organism can be provided, but there may be protocols where this is not assessed, even though it could be.

These definitions are modified from RFC 2119
(https://tools.ietf.org/html/rfc2119)

1) Cells at the start of procedure

This section describes the characteristics and state of the cells used in the procedure prior to any form of cell manipulation processes such as cell expansion and/or differentiation.

a) Essential information about the donor

i) Species and strain
The taxonomy of the organism from which the cells originated. You **must** use names according to the NCBI Taxonomy[5]. If the strain of the species is known, you **should** indicate this.

EXAMPLE: Homo sapiens/human; Mus musculus, Rag$^{-/-}\gamma_c^-$ (B6, H-2b)

ii) Characteristics of the organism
Include information about the organism from which the cells originated that is not adequately described by the species/strain information. This **may** include details of their health, age, sex or any treatments or environmental conditions to which they have been exposed to (e.g. medication). You **may** also include information that is specific to your laboratory, such as an individual identifier number. If you have purchased experimental animals (e.g. BALB/c mice) or tissues (e.g. human bone marrow) you **should** indicate the source of purchase.

EXAMPLE: healthy/ volunteer/ male/ 6-weeks-old/ male/ BALB/c mice/ purchased from Charles River (Margate England)

b) Source of cell material

The organ, tissue or fluid from which the cells have been isolated **must** be stated. If you use a blood product you **should** state the product and the source (e.g. hospital department, blood bank) from where it was obtained. You **should** use terminology from Uberon[6], or the Foundational Model of Anatomy[7]. You **should** also indicate the quantity of the sample by mass or volume, and, if applicable, which anti-coagulant was used. Additional details **must** be included if the source material was derived from cryopreserved samples (e.g., umbilical cord blood). This would include the methods and duration of storage and initial cell counts. The statement on use/ ethics committee approval/ written informed consent **MUST** be included.

EXAMPLE: Apheresis / buffy coat / bone marrow aspirate/ peripheral blood, Sanquin blood supply; 250 ml; EDTA

c) Cell separation process

i) Cell handling and labelling
The methodology used to extract the cells from the source material **must** be stated. You **should** also indicate the time between cell material retrieval and start of the isolation process. You **should** indicate how the tissue was kept during this time, including the temperature and you **may** indicate the container and fluid. You **must** indicate cell labelling procedures including characteristics and source of labelling buffers and reagents. Other details, such as cell suspension volume and concentration, incubation temperature and washing steps **should** be included.

EXAMPLE: Apheresis products were stored overnight at 4°C; Tregs were enriched by magnetic-activated cell sorting (MACS® Technology); Cells were labelled with anti-CD8 coated magnetic beads (CliniMACS® CD8 Reagent, Miltenyi Biotec) in 95 mL of PBS containing 1 mmol/L EDTA and 0.5% human albumin (PBS/EDTA buffer, Miltenyi Biotec) for 30 min at room temperature on an orbital shaker.

ii) Cell separation equipment and process
The equipment (e.g. AutoMACS®, CliniMACS®, Aria III™ Fluorescence Activated Cell Sorter) and process used to enrich for the cells of interest **should** be stated. The presence of the target population in the

starting material should be described.

EXAMPLE: Anti-CD8 bead labelled cells were resuspended in 100 mL of PBS/EDTA/0.5% HA. $CD8^+$ cells were depleted with the use of the 2.1 depletion program on the CliniMACS® Cell Separation Device (Miltenyi Biotec).

d) Phenotype

Characteristics of the cells that have been isolated **should** be described and how this has been determined. Where only a proportion of cells in the population display a characteristic, you **should** indicate the percentage.

i) Cell surface and intracellular markers

Identifying molecules that are, or are not, expressed by the cells on their surface or intracellularly is useful. You **should** describe: 1) what you measured, 2) the methodology used for the measurement (including information on reagents; if using mAbs, information on clonotype, conjugate and manufacturer **must** be provided, 3) whether the cells received a stimulus and for how long before the measurement was carried out, and 4) the method used to set marker or population positivity (e.g., fluorescence minus one method). You **should** use cluster of differentiation (CD) names when available (e.g., use CD62L instead of the alternative name L-selectin) – a full list of regularly updated CD numbers can be found on the website run by the HCDM[8] (human cell differentiation molecules). Otherwise, you **may** use databases e.g. Uniprot[9] for proteins and ChEBI[10] for non-protein organic molecules.

EXAMPLE: FOXP3 (PE-Cy7, clone PCH101, eBioscience) expression was measured directly after cell isolation by intracellular staining using the Foxp3/Transcription Factor Staining Buffer Set from eBioscience. Percentage of $CD4^+CD25^{high}CD127^{-/low}FOXP3^+lin^-doublet^-$ Treg cells was determined by flow cytometry (FACS Canto II™, Becton Dickinson). After the isolation 98.0% (median, range 97–99.5%) of the cells presented this phenotype.

ii) Secreted molecules

Molecules that are, or are not, secreted by the cells are useful to identify. These include cytokines (e.g. IL-10) and other soluble mediators. You **should** describe: 1) what you measured, 2) If using Abs, clone, conjugate and source of all antibodies and reagents used **must** be provided, 3) the methodology used for measurement, 4) cell density/ml of medium and plastic ware (e.g. 96w round/flat bottom), 5) when supernatant was collected for cytokine concentration measurement, and 6) whether the cells received a stimulus and for how long before the measurement was carried out.

EXAMPLE: IFN-γ; ELISA; supernatant after 24 hours of unstimulated cell culture.

iii) Epigenetic modifications

Epigenetic modification relevant to the characteristics **should** be described if determined. Method of detection DNA demethylation **should** be clearly described.

EXAMPLE: The mean percentage of demethylated TSDR of the foxp3 gene in the Treg population was 7% (Epiontis, Berlin, Germany).

iv) Specificity

Polyclonal or antigen-specific, especially genetic modifications to manipulate specificity **should** be described. You **should** describe: 1) what is the specificity of the cells, 2) the methodology used to obtain the specificity, and 3) the methodology used to confirm the specificity. To describe the specificity of your cells, you should use cluster of differentiation (CD) names when available (e.g. use CD19 instead of the alternative name B4) – a full list of regularly updated CD numbers can be found on the website run by the HCDM8 (human cell differentiation molecules). Otherwise, you may use databases e.g. http://hla.alleles.org for HLA alleles, Uniprot9 for proteins and ChEBI10 for non-protein organic molecules describing the targets for your cells.

EXAMPLE: HLA-A2-specific CAR (A2-CAR) T regulatory cells were generated with lentiviral vectors encoding an HLA-A2–specific CAR by cloning and sequencing the heavy- and light-chain variable regions of the mAb and fusing the resulting scFv to portions of CD8, CD28, and CD3ζ in a second-generation CAR structure. Tetramers made from HLA-A2 were used to confirm the specificity of binding the cells to HLA-A2.

e) Cell numbers

i) Absolute cell number
You **should** indicate the total number of cells present after extraction, and how they have been counted.

EXAMPLE: 980 x 10^6 cells as determined by Coulter counting

ii) Viability
You **should** indicate the percentage of cells that are alive, and how this has been determined. The percentage of apoptotic cells should be stated if determined (indicate whether the starting material is fresh or frozen).

EXAMPLE: 95% viability as determined by trypan blue exclusion. 5% of $CD3^+$ T-cells had a phenotype indicating early apoptosis (7-AAD^-, $AnnexinV^+$) as measured by flow cytometry.

2) Expansion/differentiation

The section describes the protocol that has been used for expansion/differentiation of the isolated cells described in the previous section (section 1). This process will hereafter be referred to as the expansion/differentiation process.

a) Pre-culture conditions
The conditions under which the cells are kept after isolation but before starting the expansion/differentiation process (the fluid and type of container they are kept in, and at what temperature) **should** be described. The indication whether the starting material is fresh or thawed **must** be provided. You **should** also indicate the length of time between cell extraction and start of the expansion/differentiation process.

EXAMPLE: Isolated cells were placed in PBS with1% human serum albumin in a Falcon tube and kept at room temperature for up to 30 min before starting the culture.

b) Culture conditions
The conditions under which the cells are kept during the expansion/differentiation process **should** be stated.

i) Cell number
The number of cells used for the expansion/differentiation process **should** be stated, if different from numbers stated in section 1e)*i).*

EXAMPLE: In total 5 x 10^6 cells were put into culture

ii) Cell concentration
The concentration of cells in the medium at the start of and throughout the expansion/differentiation process **should** be stated as cells/ml.

EXAMPLE: Cells were put into culture at a concentration of 1 x 10^6 cells/ml

iii) Culture medium
The medium in which the cells are grown **must** be described, including its source, and whether it has any additives (e.g. antibiotics, inactivated serum), excluding the stimuli that are described later. If you use more than one type of medium, or refresh the medium during the culture, then you **should** describe that here.

EXAMPLE: X-VIVO15 (Lonza) supplemented with5% human male type AB-serum (Sigma)

iv) Culture container
The physical container in which the culture is carried out. This can include tissue culture plates, tissue culture bags or flasks. You **should** state the type of container, size and manufacturer. You **should** also indicate the total cell culture volume per container or well, as well as the total number of containers used.

EXAMPLE: 20 ml of medium in a 100 ml MACS GMP Cell Differentiation bag (Miltenyi Biotec); 1 bag

v) Culture environment
Describe the physical environment in which the cells are kept during the expansion/differentiation process. This **should** include the temperature and CO_2 concentration. You **should** note whether medium has been pre-warmed. You **may** describe the equipment used to maintain the culture environment.

EXAMPLE: 37 ℃, 5% CO_2; Medium was pre-warmed to 37 ℃; Sanyo CO_2 incubator

c) Expansion/Differentiation protocol
The protocol that is used to expand/differentiate the cells **should** be described. This **must** include the type and source of cytokine(s) or other agent(s) added into the medium, and at what time point and concentration **should** be included. You **should** also state the total length of the culture period as well as the rounds of stimulation, rounds of culture change and the number of cell passages.

EXAMPLE: Rapamycin (final concentration of 100nM; Rapamune®, Pfizer) was added on day 0, 2, 5, 7 and 9. IL-2 (final concentration of 500IU/mL; Proleukin®, Novartis) was added on day 2, 5, 7 and 9. Cells were harvested on day 12.

d) Stimulus
It **should** be stated whether the cells are expanded/differentiated polyclonally or in an antigen-specific manner or against an alloantigen. The protein(s), antibody(ies), accessory cells or other preparation(s) (e.g. antigen-presenting cells; APCs) with which the cells are stimulated **must** be named. You **must** describe the source of the preparation, concentration and time point(s) at which it/they are added to the cell culture. Restimulation conditions, if any, should also be stated.

EXAMPLE:
Cells were stimulated with CD3/CD28 MACS GMP ExpAct Treg Beads (Miltenyi Biotec) at a 4:1 bead:cell ratio. Cells were stimulated with CD40-activated allogeneic B cells (30 Gy-irradiated) at a ratio of 10 B cells per nTreg cell.

e) Storage
The conditions in which the cells are kept after completion of the expansion/differentiation process, but before being used in any subsequent experimental assay or treatment **should** be described. You **should** indicate the fluid and temperature in/at what the cells are being kept, as well as the length of time. You **should** indicate if cells are being frozen, and give details on the freezing and thawing procedures, including cell recovery and viability after thawing. You **should** also indicate if cells are taken out of their culture environment for any length of time during the expansion/differentiation process (e.g. if cells are frozen before completion of this process, with the aim to resume it at a later date).

EXAMPLE: Cells were kept in PBS 1% human serum albumin (Sigma) in a 50 ml Falcon tube at room temperature for a maximum of 2 hours; Cells were frozen in FCS/10% DMSO.

3) Cells after expansion/differentiation

This section describes the characteristics and state of the cells at the end of the expansion/differentiation process described in the previous section (Section 2).

a) Phenotype

Characteristics of the cells at the end of their expansion/differentiation, including their specificity and purity (e.g. as % of target cells) **must** be described. Where only a proportion of cells in the population display a characteristic, you **should** indicate the percentage. You **should** report on the stability of the phenotype and how you determined this. It **should** be indicated if the phenotype of the cells post-expansion was determined using fresh viable cells, or rather after a freeze-thaw cycle in a batched analysis.

i) Cell surface and intracellular markers

A number of phenotypic markers help to define the Treg cellular phenotype and specificity and are associated with distinct expression levels of surface and intracellular proteins. These markers are often characteristic of the transcriptional program of a cellular lineage and provide important information regarding the phenotypic stability and function of resulting cell products. You **should** describe: 1) what you measured, 2) the methodology used for measurement (including information on reagents; if using mAbs, information on clonotype, conjugate and manufacturer **must** be provided, 3) whether the cells received a stimulus and for how long before the measurement was carried out, and 4) the method used to set marker or population positivity (e.g., fluorescence minus one method). You **should** use cluster of differentiation (CD) names when available (e.g. use CD127 instead of the alternative name IL-7Rα) – a full list of regularly updated CD numbers can be found on the website run by the HCDM[8] (human cell differentiation molecules). Otherwise, you **may** use databases e.g. http://hla.alleles.org for HLA alleles, Uniprot[9] for proteins and ChEBI[10] for non-protein organic molecules.

EXAMPLE: Intracellular IFN-γ and IL-17 expression was measured by flow cytometry after 4 h incubation with 20 ng/ml PMA and 1 µg/ml Ionomycin in the presence of 1µl/ml GolgiPlug™ using the BD Cytofix/Cytoperm™ buffer set.

ii) Secreted molecules

Indicate molecules that are, or are not, secreted by the cells. These include cytokines (e.g. IL-10) and other soluble mediators. You **should** describe: 1) what you measured, 2) if using mAbs, clone, conjugate and source of all antibodies and reagents used **must** be provided, 3) the methodology used for the measurement, 4) cell density/ml of medium and plastic ware (e.g. 96w round/flat bottom), 5) when supernatant was collected for cytokine concentration measurement, and 6) whether the cells received a stimulus and for how long before the measurement was carried out.

EXAMPLE: Soluble IFN-γ, TNF-α, IL-17 and IL-10 were measured in the cell culture supernatant at a cell density of 1x10^6 cells/ml by ELISA according to the manufacturers' instruction.

iii) Epigenetic modifications

Epigenetic modification relevant to the characteristics **should** be described if determined. Method of detection DNA demethylation **should** be clearly described.

EXAMPLE: The mean percentage of demethylated TSDR of the foxp3 gene in the Treg population was 97% (Epiontis, Berlin, Germany).

b) Functional assay
You **should** describe any characteristic of the cells that has been measured by a functional assay (type of assays). This could either be the response of the cells to some stimulus or the behaviour of other biological entities after exposure to the cells. There should be a clear indication of how the percentage of suppression was calculated (i.e. include formula). Whenever accessory cells such as responder cells are included in the assay, source and phenotype should be described. Behaviour such as expression/production of molecules (described in Section 3a) does not need to be included.

EXAMPLE: Proliferation-based suppression assay using CFSE labelled autologous $CD4^{+}CD25^{-}$ responder cells; IFN-γ based suppression assay

c) Cell numbers

i) Absolute cell number
You **must** indicate the total number of cells present at the end of the expansion/differentiation process, and how they have been counted and fold expansion **should** be included.

EXAMPLE: Cell numbers were microscopically determined using C-Chip disposable counting chambers from NanoEnTek and fold expansion to day 0 was calculated.

ii) Viability
You **must** indicate the percentage of cells that are alive, and how this has been determined **should** be included.

EXAMPLE: 83% viability as determined by trypan blue exclusion

d) Dosing
Whenever cells are transferred into an organism, details about dosing **must** be given. For clinical applications, information on the vehicle (solvent/medium) as well as intermediate components (trace amounts possible) **must** be given.

EXAMPLE: A single dose of $1x10^{7}$ total nucleated cells per kilogram of body weight in 50 ml 0.9% NaCl was transfused iv.

e) Quality control
If the cells were produced for a clinical trial, you **must** describe release criteria and any methods used to determine sterility, specificity, purity and quality of the product.

4) About the protocol

In this section, we describe the general features about the protocol as a whole.

a) Regulatory authority
Information about whether the protocol being used has been validated or quality-controlled to standards agreed to by an external regulatory authority **must** be stated. You **should** state the name of this authority. Also you **should** state whether the protocol follows Good Manufacturing Practice (GMP).

EXAMPLE: Medicines and Health Regulatory Authority (MHRA)

b) Purpose
You **must** describe the overall purpose of the production of the cells.

EXAMPLE: Prevention of transplant rejection; Treatment of patients affected by Crohns' disease.

c) The relationship between the organism of origin of the cells and the target organism
You **must** state if the cell product is autologous/allogeneic/xenogeneic/syngeneic to the recipient.

EXAMPLE: Patients receiving allogeneic kidney transplants and autologous Tregs. B6 mice receiving allogeneic (BALB/c xB6) heart transplants and syngeneic (B6) Tregs.

d) Contact details
You **must** provide the name and contact information of the corresponding author(s).

e) Citation
You **should** add information that your paper was written in accordance with the MITreg reporting guidelines.

Footnotes
1) http://flowcyt.sourceforge.net/miflowcyt/
2) http://fged.org/projects/miame/
3) http://miataproject.org
4) https://doi.org/10.7717/peerj.2300
5) http://www.ncbi.nlm.nih.gov/taxonomy/
6) http://www.uberon.org
7) http://fme.biostr.washington.edu/FME
8) http://www.hcdm.org/
9) http://www.uniprot.org/
10) https://www.ebi.ac.uk/chebi/